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Extended x-ray absorption fine structure of copper in cytochrome *c* oxidase: Direct evidence for copper–sulfur ligation

(cytochrome aa_3/copper sites/sulfur ligation/x-ray absorption spectroscopy/x-ray fluorescence detection)

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ABSTRACT The copper x-ray fluorescence excitation spectrum of cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) has been recorded in the 245–270 K range. The beat pattern observed in the extended x-ray absorption fine structure can be accounted for only by postulating a combination of sulfur and nitrogen (or oxygen) ligands to the copper. The average Cu–S distance is 2.27 ± 0.02 Å and the average Cu–N (or Cu–O) distance is 1.97 ± 0.02 Å. The amplitudes require *ca.* 1–1.5 sulfurs and 2 nitrogens (or oxygens) per copper. The distribution of sulfur ligands between Cu_A and Cu_B sites is not known, although there is some evidence that two sulfur atoms are bound to Cu_A.

As the terminal enzyme of the mitochondrial respiratory chain, cytochrome *c* oxidase (EC 1.9.3.1) catalyzes the reduction of dioxygen to water (1). The mammalian enzyme is a large (*ca.* 150,000 dalton) membrane-associated protein complex containing four distinct active sites: two heme irons (cytochrome *a* and cytochrome a_3) and two copper atoms (here designated Cu_A and Cu_B) per minimal functional unit. Detailed investigations concerning the nature of the copper active sites have been hampered by the lack of unique spectral contributions from these sites. A $g = 2$ signal in the EPR spectrum is generally believed to be associated with *one* of the copper sites (Cu_A). However, the small Cu hyperfine splitting (2) and unusual *g* values associated with this signal have led some to consider this a Cu(I)–sulfur radical site (3–6). EPR signals associated with the Cu_B site in certain enzyme derivatives (Cu_B is EPR-silent in the native enzyme) have been reported by two groups, and it has been concluded that Cu_B is not a blue (or type 1) copper (7–8). Clearly, further studies aimed at structural elucidation of both copper sites in cytochrome *c* oxidase are needed, and with this goal in mind we have performed x-ray absorption experiments using synchrotron radiation and a fluorescence detection apparatus.

Previous x-ray absorption work on cytochrome *c* oxidase copper has been limited to studies of the absorption edge region. Early experiments were performed by Hu, Chan, and Brown, who concluded from the shape and position of the edge that one copper of the resting enzyme was actually in the reduced [Cu(I)] form (9). Hu *et al.*, also investigated the effects of cyanide treatment on the iron and copper absorption edges, finding no change in the copper edge of either the oxidized or the dithionite-reduced sample (10). Later work was done by Powers and coworkers (11), who disputed the previous Cu(I) assignment and attributed some findings of the work by Hu *et al.*, to adventitious copper and photoreduction. They also reported copper edges for the mixed valence-CO and fully reduced oxidation levels and came to the conclusion that Cu_B is a type 1 copper similar to that of stellacyanin. The designation of the Cu_B site as type 1, however, does not accord with the results of other chemical and spectroscopic experiments (7, 8, 12). Furthermore, Beinert *et al.* (13) have presented spectroscopic data that show that the contribution of Cu_B to the absorbance at *ca.* 830 nm cannot be as large as suggested by Powers *et al.* (11).

The determination of structural details from absorption edges alone is a very difficult task. For this reason we have extended the data collection and analysis to the extended x-ray absorption fine structure (EXAFS) region beyond the copper edge. Apart from its capacity for determining the distances and numbers of neighboring atoms, EXAFS is also useful for distinguishing between certain types of atoms (14). In this regard, the dramatic differences between sulfur and nitrogen (or oxygen) phase shifts have been theoretically (15) and experimentally (16) documented and exploited in the characterization of molybdenum (17, 18; S.P.C., unpublished observations), copper, (19, 20), and iron proteins (21).

The question of the oxidation states of the coppers in resting state cytochrome *c* oxidase will not be addressed in the present communication. Instead, we will direct our attention to those aspects of the copper site structure that are revealed unambiguously from an EXAFS analysis, namely, the identification of ligand donor atoms in the inner coordination spheres of the coppers. In this paper we show that the Cu EXAFS may be analyzed as the sum of Cu–S and Cu–N (or Cu–O) interactions, and the results of our analysis are discussed in light of previous absorption edge and EPR results.

MATERIALS AND METHODS

Sample Preparation and Handling. All cytochrome *c* oxidase samples were prepared by procedure 1 of ref. 22. For EXAFS measurements, the samples were placed in small Lucite sample cells as solutions *ca.* 2 mM in copper. The sample cells were designed to fit into a standard-bore EPR cavity dewar in order to avoid positioning problems associated with wide-bore EPR cavities (23). EXAFS data were recorded with samples in a helium atmosphere on a stage cooled by circulation of nitrogen gas cooled by liquid nitrogen. After data collection, the samples were cooled to *ca.* −50°C while still under irradiation to trap the samples in their irradiated state. The samples were then kept at dry ice temperature until the EPR and optical reflectance spectra could be recorded (at cryogenic temperatures).

Data Collection. The spectra were recorded at the Stanford Synchrotron Radiation Laboratory, using a multi-element fluorescence detection system to be described separately (24). The signals from the scintillation detectors were weighted directly by the square of the signal-to-noise ratio [$(S/N)^2$] and inversely by total counts, and were then averaged and ratioed with the incident intensity recorded by a nitrogen-filled ionization

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Abbreviation: EXAFS, extended x-ray absorption fine structure.

chamber. The resulting signal contains a contribution proportional to the copper x-ray absorption (25) superimposed upon a background due primarily to elastic scattering. The S/N ratio was enhanced by reducing the scattering background with 10 or 20 μm nickel filters (26). The final EXAFS spectra from two typical samples of resting state (fully oxidized) cytochrome c oxidase are shown in Fig. 2. The top spectrum represents the average of 16 scans recorded on the wiggler line (beam line IV) at 2.0 GeV (at ca. 12 mA electron current) using a Si[220] crystal monochromator. These data were recorded while the sample was maintained at ca. −5°C. The bottom spectrum represents the average of 17 scans recorded on beam line I-5 (a non-wiggler line) at 3.0 GeV (under dedicated operation with ca. 100 mA electron current), using a similar Si[220] crystal monochromator. These data were recorded at ca. −27°C. The average incident photon fluxes under both sets of conditions were ca. 10^9–10^{10} photons per second and each sample remained in the beam for ca. 16 hr.

Data Analysis. The EXAFS was extracted by using a cubic spline with a spline point at 9400 eV, normalized to the edge jump and adjusted for the atomic falloff by using the Victoreen formula (27). The spectra were smoothed by convolution with a Gaussian (0.25 $Å^{-1}$, full width $1/e$). The E_0 used for calculating k was 9000 eV.

Curve-fitting analysis was performed with a program written by Eccles (19), and modified to fit spectra according to the formula:

$$\chi(k) = \sum_s \frac{N_s A_{as}(k)}{k R_{as}^2} \sin[2kR_{as} + \alpha_{as}(k)].$$

In this expression, N_s is the number of scatterers (s) at distance R_{as} from the absorber (a). The amplitude $A_{as}(k)$, which has been parameterized as $c_0 \exp(c_1 k^2) k^{c_2}$, represents the cumulative effect of electron–atom backscattering amplitude, Debye–Waller factor, elastic yield factors, and other effects. Similarly, $\alpha_{as}(k)$, parameterized in this case as $a_0 + a_1 k + a_2 k^{-1}$, is the sum of phase shifts due to absorber and scatterer. The optimization proceeded by minimizing:

$$\sum_{k=4}^{12} [\chi(k) - \chi_{calc}(k)]^2 \cdot k^6.$$

The curve-fitting analysis used phase shifts and amplitudes previously developed for hemocyanin (19), blue copper (20), and iron (21) EXAFS studies.

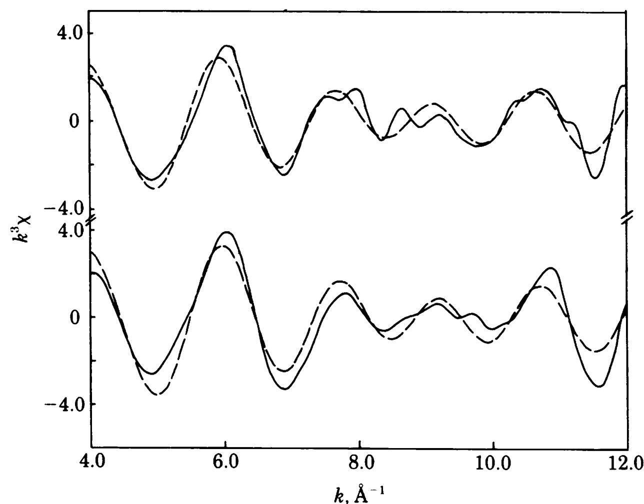

FIG. 2. Copper EXAFS of two samples of resting state (fully oxidized) cytochrome c oxidase. The top spectrum was recorded on a sample at −5°C under nondedicated conditions on the wiggler line. The bottom spectrum was recorded on a sample at −27°C under dedicated conditions on line I-5. In each, the solid line is the smoothed data and the broken line is the fit. The differences between the two spectra are indicative of the noise in the data.

RESULTS

A typical x-ray fluorescence excitation spectrum for cytochrome c oxidase is illustrated in Fig. 1. There are two strong maxima visible in the spectra, after which the EXAFS damps out extremely rapidly. The EXAFS data and fits are shown in Fig. 2. A Fourier transform of the data is presented in Fig. 3.

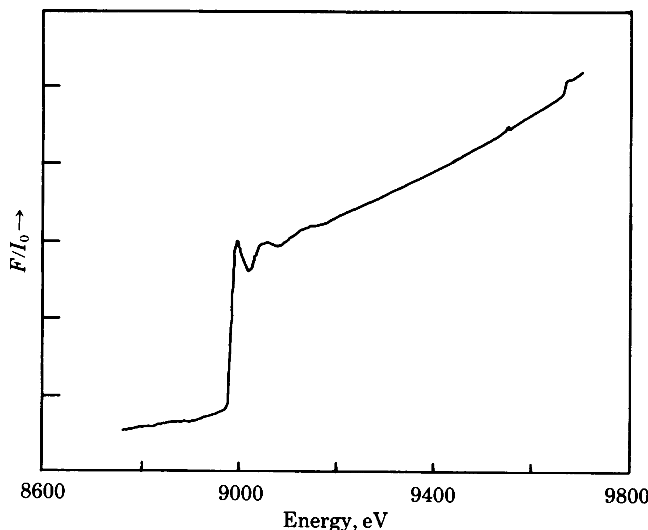

FIG. 1. The x-ray fluorescence excitation spectrum of the copper in cytochrome c oxidase. The upward-sloping background is due primarily to elastic scattering by water and protein. There is a glitch at 9550 eV because of a sudden dip in I_0 at that point. The amount of zinc present is more than is indicated by the edge at 9660 eV, because most of the zinc fluorescence is absorbed by the nickel filters.

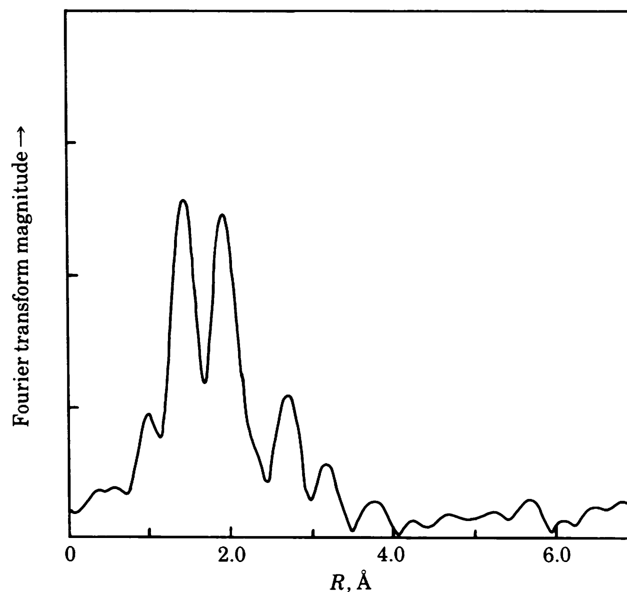

FIG. 3. The Fourier transform (3.5–12.5 $Å^{-1}$, k^3 weighting) of the bottom spectrum of Fig. 2. Because of the phase shifts involved, the true distances are approximately 0.3–0.4 Å longer than indicated by the abscissa.

The curve-fitting results for the data in Fig. 2 indicate an average of 1.8 nitrogens (or oxygens) at 1.97 ± 0.02 Å and 1.4 sulfurs at 2.27 ± 0.02 Å (±SD). The presence of at least two quite different copper–ligand interactions is required by the beating of the EXAFS and is shown clearly by the Fourier transform. The assignments as Cu–N (or Cu–O) and Cu–S, respectively, depend on the relative uniqueness of the Cu–N (O) and Cu–S total phase shifts. In fits such as these a single Cu–S interaction could be mimicked only by a component representing roughly −2 nitrogens or oxygens. All attempts to include a short Cu–S component such as previously observed for oxidized blue copper proteins (20) were unsuccessful. Examination of the fits and consideration of the expected types of copper ligation make it clear that other components must be added to completely model the EXAFS. For example, other minor Cu–N (or Cu–O) components might well be present. Furthermore, a Cu–C interaction, similar to the interactions observed in the Cu EXAFS of hemocyanin and blue copper proteins, seems indicated by the Fourier transform. However, we have decided to await the collection of data with better S/N before attempting a complete analysis.

DISCUSSION

There is a difference of opinion concerning the extent of photoreduction of cytochrome *c* oxidase during x-irradiation experiments (11, 23, 28). For this reason, we have taken great care to assay the frozen samples by both EPR and optical reflectance spectroscopy after data collection. We have noticed up to *ca.* 30% reduction in some samples (the data from which are *not* included in the fits discussed here) and thus find that partial reduction of cytochrome *c* oxidase samples under synchrotron irradiation may occur. Of the two samples used in this study, however, one showed *no* evidence of reduction, while the other showed a small amount of reduction (*ca.* 12% reduction in the g3 signal and *ca.* 20% reduction in the g2 signal). Thus, we are confident that the data presented here are for fully oxidized enzyme.

The Cu–N (or Cu–O) distance of 1.97 Å is in the range expected for Cu–N bond lengths, and the amplitude corresponds to at least 2 N (or O) per copper. The data provide evidence for a Cu–S component at 2.27 Å, a distance that is substantially longer than the 2.12- to 2.14-Å Cu–S(cysteine) bond length in oxidized blue copper proteins (20). This 2.27-Å Cu–S distance is close to that observed for Cu–S(cysteine) in reduced blue copper proteins, 2.22–2.25 Å (K. O. Hodgson, personal communication), but it is also in the same range as the Cu–S bond lengths observed in square planar Cu(II) complexes (29, 30). The amplitude of the Cu–S component corresponds to two or three sulfurs per two coppers. From the EXAFS alone we cannot say whether sulfur is part of the coordination sphere of each copper or whether all of the sulfur is ligated to only a single type of copper. Finally, we cannot exclude other minor Cu–N contributions, or longer distance (>2.4-Å) Cu–S interactions.

On the basis of the combination of our EXAFS results with edge and EPR data, it is not unreasonable to suggest that at least two sulfurs be assigned to the Cu_A site (ref. 6; T. H. Stevens and S. I. Chan, personal communication). Powers *et al.* (11) have shown with their absorption edge work that Cu_A is significantly more covalent than Cu_B. This conclusion stems from the observation of a large edge shift in going from the oxidized to the mixed valence-CO state, during which Cu_B is presumably reduced, and a smaller shift in going from the mixed valence-CO state to the fully reduced state, during which Cu_A is reduced. It is well established that edge shifts between oxidation states decrease as the number of sulfur ligands increases. If only one sulfur were present at each copper site, an alternative explanation for the difference in edge shifts would have to be found, whereas assignment of two sulfurs to Cu_A accommodates the edge results nicely. Furthermore, the electron–nuclear double resonance experiments of Hoffman *et al.* (31) may be interpreted satisfactorily by assuming that two sulfur donors are bound to Cu_A.

In summary, our EXAFS analysis has established sulfur ligation to copper in cytochrome *c* oxidase. Elucidation of the details of the distribution of sulfurs between the two coppers must await further measurements, but a reasonable working hypothesis assigns two sulfurs to one copper (Cu_A). Once data for mixed-valence and fully reduced forms of cytochrome *c* oxidase are in hand, we will have an independent means of assigning the sulfurs to particular coppers. Thus, if all sulfurs reside on Cu_A, we would expect no change in the Cu–S bond length upon going to the mixed valence-CO state, and then an increase in this distance upon complete reduction of the enzyme.

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